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A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*

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ABSTRACT **Plant lipoxygenases are thought to be involved in the biosynthesis of lipid-derived signaling molecules. The potential involvement of a specific *Arabidopsis thaliana* lipoxygenase isozyme, LOX2, in the biosynthesis of the plant growth regulators jasmonic acid (JA) and abscisic acid was investigated. Our characterization of LOX2 indicates that the protein is targeted to chloroplasts. The physiological role of this chloroplast lipoxygenase was analyzed in transgenic plants where cosuppression reduced LOX2 accumulation. The reduction in LOX2 levels caused no obvious changes in plant growth or in the accumulation of abscisic acid. However, the wound-induced accumulation of JA observed in control plants was absent in leaves of transgenic plants that lacked LOX2. Thus, LOX2 is required for the wound-induced synthesis of the plant growth regulator JA in leaves. We also examined the expression of a wound- and JA-inducible *Arabidopsis* gene, *vsp*, in transgenic and control plants. Leaves of transgenic plants lacking LOX2 accumulated less vsp mRNA than did control leaves in response to wounding. This result suggests that wound-induced JA (or some other LOX2-requiring component of the wound response pathway) is involved in the wound-induced regulation of this gene.**

The enzyme lipoxygenase (LOX; EC 1.13.11.12), which plays a key role in lipid-based signaling systems in mammals (1), has been postulated to have an analogous role in plants. LOX expression in plants is regulated throughout development and in response to wounding and water deficit (2–4). LOX expression and activity are also modulated by exposure to pathogens (5, 6). This regulation of LOX expression may be important for providing signaling molecules that mediate the plant's developmental and defensive responses to varying growth conditions. Plant LOXs catalyze the hydroperoxidation of certain C-18 unsaturated fatty acids, providing intermediates for a number of specific products, including the plant growth regulator jasmonic acid (JA) (7). JA accumulates in wounded tissue and in response to fungal elicitors (8, 9) and has been proposed to modulate the wound-induced expression of several genes (10). A possible role for LOX activity in the synthesis of the plant growth regulator abscisic acid (ABA), which accumulates in plants exposed to water deficit, has also been suggested (7, 11).

To evaluate the significance of LOX regulation for modulating the production of particular signaling molecules, it is necessary to identify physiological roles for individual LOX isozymes. We have been pursuing this by studying the regulation, localization, and function of one LOX gene product, LOX2, from *Arabidopsis thaliana*. The gene *Lox2* differs significantly both in deduced amino acid sequence and in expression pattern from the other known *Arabidopsis* LOX gene, *Lox1*, suggesting that the corresponding enzymes may have distinct roles within the plant (3, 12). We have used a transgenic approach to modify *Lox2* expression in *Arabidopsis* and report the consequences of reduced *Lox2* expression for plant growth and for the synthesis of JA and ABA.

MATERIALS AND METHODS

Chloroplast Isolation and Protein Uptake. Pea chloroplasts were isolated from green tissue by the method of Baumgartner *et al.* (13). Radioactively labeled LOX2 was synthesized from *in vitro*-transcribed mRNA by using rabbit reticulocyte lysate (Promega). Chloroplast uptake incubations were done for 30 min in the light in 200 μl containing 5×10^7 pea chloroplasts, 15 μl of *in vitro* translation mixture, 8 mM unlabeled methionine, 50 mM Hepes·KOH (pH 8), and 0.3 M sorbitol. After uptake, chloroplasts were washed and one sample was treated with protease (thermolysin, 100 μg/ml) for 30 min on ice. Samples were analyzed by SDS/PAGE (7.5% gel) and autoradiography.

Generation and Preliminary Analysis of Transgenic Plants. The vector pRTL (14), which contains the 35S promoter from cauliflower mosaic virus, was modified by replacing the *Eco*RI–*Sma* I fragment with an *Eco*RI adaptor. Colonies resulting from insertion of the *Lox2* cDNA *Eco*RI fragment (3) into this modified vector were screened to check insert orientation (sense clones had the 5′ end of the cDNA adjacent to the 35S promoter). *Hin*dIII promoter–gene construct fragments from each selected clone were inserted into the *Hin*dIII site of the plant transformation vector PGA482 (15). Sense and antisense *Lox2* constructs in PGA482 were transformed into *Agrobacterium tumefaciens* LBA4404, and these cell lines were used to transform *Arabidopsis* (ecotype Columbia) as described by Valvekens *et al.* (16). Plants arising from callus were designated T_0; seeds from T_0 and plants growing from them were designated T_1. Individual T_1 plants were grown, and some of the resulting T_2 seeds were sown on medium containing kanamycin at 50 μg/ml. T_2 seed lots that gave rise to 100% kanamycin-resistant or kanamycin-sensitive plantlets in this screening were assumed to result from T_1 plants that were homozygous for the presence or absence, respectively, of transferred DNA (T-DNA), and were designated T for transgenic or C for control.

Nucleic Acid Analysis. RNA was isolated and analyzed as described (2). Leaf and root RNAs were from plants grown in culture (3); inflorescence RNAs were from plants grown in soil. Genomic DNA was isolated by the protocol of Dellaporta *et al.* (17) from plants grown in culture. A random-primer-labeled 2.1-kb *Sac* I fragment of the *Lox2* cDNA (3) was used as the *Lox2* probe.

Antibody Production and Protein Analysis. The 2.1-kb *Sac* I fragment of the *Lox2* cDNA (3) was inserted into the *Sac* I site of the expression vector pQE30 (Qiagen). Clones with this insert in the correct orientation resulted in the expression of a partial LOX2 protein in which the 218 amino acids from the N terminus were replaced by 14 amino acids from the vector,

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Abbreviations: LOX, lipoxygenase; JA, jasmonic acid; ABA, abscisic acid.

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including a 6-His motif used for fusion protein purification. Fusion protein expressed from this construct was isolated as described by the vector manufacturer's protocol (Qiagen), with a SDS/PAGE fractionation step added to provide additional purification. The resulting protein was used to inoculate rabbits for antibody production. For Western blot analysis, leaves or inflorescences were harvested from plants grown in soil, ground in a small volume of 50 mM Tris·HCl (pH 7.5), and centrifuged briefly. *Arabidopsis* chloroplasts were isolated by standard protocols for other species (13). Leaf or inflorescence soluble proteins or chloroplast total proteins were separated by SDS/PAGE, followed by electrotransfer to Immobilon-P (Millipore). Immune serum raised against the LOX2 fusion protein was used at a 1:5000 dilution with chemiluminescent detection (ECL, Amersham) to identify reactive proteins on the blots.

Plant Stress Treatments. *Arabidopsis* plants were grown in 11-h light/13-h dark cycles for 31–34 days to generate large rosettes. For wound experiments, all accessible leaves of an individual plant were wounded one to three times with pressure from a hemostat, and plants were incubated in the light for 4 h prior to harvest. Only those leaves that had been wounded were collected, while leaves from unwounded plants were harvested at 0 or 4 h for control samples. All samples were frozen in liquid N_2 immediately after harvest. For water-deficit treatments, rosettes were excised from roots and wilted by using cool air from a hair dryer until they had lost 14% of their fresh weight. Wilted and control excised rosettes were stored in closed tubes for 4 h in the dark and then frozen in liquid N_2. Zero-hour control excised rosettes were harvested with the other samples but frozen immediately. All samples were stored at −70°C until analysis.

JA Analysis. Frozen leaf tissue (3–5 g) was homogenized in 50:50 (vol/vol) acetone/methanol, and a known amount of [^{13}C]JA was added as an internal standard. After nearly complete removal of the solvents by rotary evaporation, 50 ml of 0.1 M sodium phosphate, pH 7.8/5% (wt/vol) NaCl was added, and rotary evaporation was continued for several minutes. The resulting aqueous solution was extracted twice with dichloromethane, acidified to pH ≈2.0 by the addition of HCl, and further extracted twice with hexane and then twice with dichloromethane. Under acidic conditions, JA partitioned into dichloromethane, which was collected and dried by rotary evaporation. JA was further purified by analytical HPLC and analyzed by GC-selected ion monitoring by the method of Creelman and Mullet (18).

ABA Analysis. ABA was extracted from lyophilized tissue by the method of Creelman and Zeevaart (19) except that acetonitrile was used in the place of ethanol. Quantification was performed as described by Creelman *et al.* (11), with three replicates of each treatment analyzed.

RESULTS

***In Vitro* Uptake of LOX2 into Chloroplasts.** Plant LOXs have been reported to be localized in chloroplasts, vacuoles, and the cytoplasm (7). This compartmentalization of LOX activity may be of functional significance. While the *Lox1* sequence does not encode any obvious protein targeting signals (12), the deduced amino acid sequence of *Lox2* contains a putative chloroplast transit peptide (3). Based on this, we examined whether LOX2 protein can be imported into chloroplasts. Pea chloroplasts were incubated with radioactively labeled LOX2 protein and then treated with protease to degrade proteins not imported into and protected by intact chloroplasts. As shown in Fig. 1, *in vitro*-synthesized LOX2, with a molecular mass of ≈103 kDa, is processed to a molecular mass of ≈100 kDa and protected from protease action when incubated with chloroplasts. These results demonstrate that LOX2 is a chloroplast-localized protein.

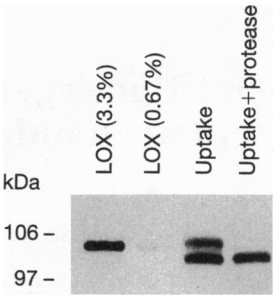

FIG. 1. LOX2 uptake into isolated pea chloroplasts. Radiolabeled LOX2 protein was incubated with isolated pea chloroplasts (uptake). One sample (uptake + protease) was further treated to degrade proteins unprotected by intact membranes. Proteins were analyzed by SDS/PAGE and autoradiography. Radiolabeled LOX2 protein at 3.3% or 0.67% of the amount added to uptake reactions was included on the gel to indicate whether LOX2 is processed during uptake and to estimate uptake efficiency.

Generation and Characterization of Transgenic Plants. To examine physiological roles for this chloroplast-localized LOX, *Arabidopsis* was transformed with sense or antisense constructs of *Lox2* cDNA behind a constitutive promoter. As part of our preliminary analysis, we examined Lox2 mRNA levels in the leaves, roots, and inflorescences of transgenic and control plants from 18 lines transformed with one of these constructs. To further characterize the consequences of transgene insertion, an antibody raised against a truncated LOX2 fusion protein was used to examine the abundance of LOX2 protein in leaves and inflorescences of a subset of these transformed lines. Five lines transformed with the sense construct had at

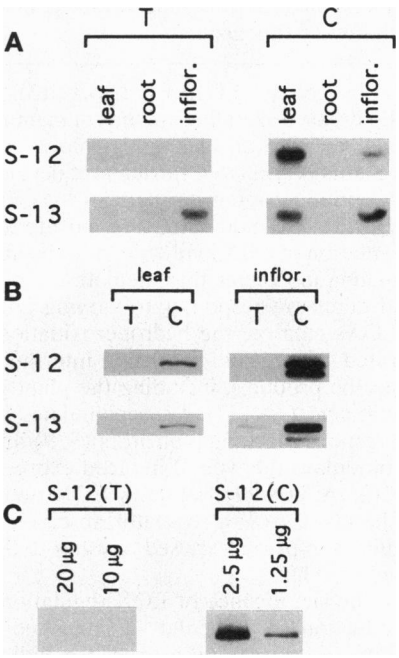

FIG. 2. Characterization of the effects of *Lox2* transgene insertion on *Lox2* expression in specific tissues of the transformed lines indicated. Transgenic (lanes T) and control (lanes C) plants of each line were examined. (*A*) Analysis of *Lox2* mRNA abundance. RNAs were isolated from leaves and roots grown in culture or from inflorescences of plants grown in soil. RNA blots were probed with a random primer-labeled *Sac* I fragment of the *Lox2* cDNA (3). (*B*) Western blot analysis of LOX2 protein abundance in leaves and inflorescences. The anti-LOX2 antibody reacts with a protein or proteins of ≈95–100 kDa. Fifteen micrograms of tissue extract per lane was loaded. (*C*) Western blot analysis of LOX2 protein abundance in extracts of isolated chloroplasts. The anti-LOX2 antibody reacts with a protein or proteins of ≈95–100 kDa. The amount of protein loaded per lane is indicated.

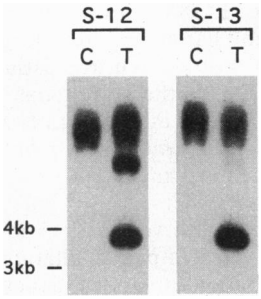

FIG. 3. Analysis of genomic DNA from control (lanes C) and transgenic (lanes T) plants of the transformed lines indicated. DNA (2 μg per lane) was digested with *Hin*dIII and fractionated on a 0.7% agarose gel. The resulting blot was probed with a random primer-labeled *Sac* I fragment of the *Lox2* cDNA (3).

least some increase in LOX2 expression in the transgenic plants compared to corresponding control plants. Three lines transformed with the sense construct and three lines transformed with the antisense construct had some decrease in LOX2 expression in transgenic plants compared to LOX2 levels detected in control plants. In four of these lines (three transformed with the sense construct and one transformed with the antisense construct), this reduction in LOX2 expression was substantial in at least one of the tissues examined.

mRNA and protein analysis of two sense construct lines with reduced LOX2 expression is shown in Fig. 2. Transgenic plants from the transformed lines S-12 and S-13 [S-12(T) and S-13(T), respectively] had greatly reduced levels of Lox2 mRNA in leaves and/or inflorescences compared to corresponding control plants [S-12(C) and S-13(C)] (Fig. 2*A*). In contrast, no substantial alterations in Lox1 mRNA levels were seen (data not shown). Presumably the previously described phenomenon of cosuppression (20) is causing the specific reduction in Lox2 expression in these plants containing sense constructs. As shown in Fig. 2*B*, the S-12 and S-13 lines also had reduced levels of LOX2 protein in the transgenic plants. The significance of the protein doublet detected by this antibody in inflorescence extracts is not clear. However, the reduction in the levels of both proteins in the transgenic plants suggests that both are products of the *Lox2* gene.

Chloroplasts were isolated from transgenic and control plants of the S-12 line to quantify the effects of transgene insertion on LOX2 accumulation. Analysis of different concentrations of S-12(T) and S-12(C) chloroplast protein extracts on the same Western blot indicated that LOX2 protein is reduced >15-fold in S-12(T) chloroplasts compared to S-12(C) chloroplasts (Fig. 2*C*). The same result was observed when total leaf extracts were examined (data not shown).

Analysis of genomic DNA from S-12 and S-13 transgenic plants indicates the presence of a 3.5-kb *Hin*dIII fragment containing the *Lox2* transgene (Fig. 3). This fragment, which is internal to the transferred T-DNA, is absent in DNA from corresponding control plants. S-12(T) genomic DNA also contains an additional *Lox2* copy on a *Hin*dIII fragment that is not the expected size. Insertion of multiple T-DNA copies at single or multiple loci and rearrangement of the T-DNA insert are known phenomena in plant transformation (21). It is not known whether the presence of more than one copy of the *Lox2* transgene is contributing to the efficient suppression of *Lox2* expression seen in S-12(T) plants.

Interestingly, the dramatic reduction in LOX2 levels in both the leaves and inflorescences of S-12(T) plants had no obvious effects on the growth or development of these plants. To further investigate the consequences of reduced LOX2 levels, we assayed the accumulation of two growth regulators, ABA and JA, in S-12(T) and S-12(C) plants.

ABA Accumulation. We have demonstrated (11) that several inhibitors of LOX activity reduce the accumulation of ABA in soybean cell cultures that have been subjected to an osmotic stress. To investigate whether there is an essential role for LOX2 in ABA accumulation in *Arabidopsis*, we assayed ABA levels in the leaves of S-12(T) and S-12(C) plants. The leaves were either untreated or subjected to a wilting treatment that has been reported to result in ABA accumulation in *Arabidopsis* (22). As indicated in Table 1, ABA levels are comparable in S-12(T) and S-12(C) leaves for all treatments tested, suggesting that LOX2 does not play an essential role in ABA accumulation in these tissues.

JA Accumulation. JA has been reported to be derived from linolenic acid via a LOX-dependent pathway (23). To examine the role of LOX2 in JA biosynthesis, JA levels in the leaves of S-12(T) and S-12(C) plants were analyzed. Because wounding has been shown to result in JA accumulation (8), JA levels were analyzed both in untreated and in wounded leaves. As shown in Fig. 4, JA levels in unwounded leaves of S-12(T) plants are comparable to those found in unwounded S-12(C) leaves, suggesting that LOX2 is not essential for maintaining JA levels under normal growth conditions. However, the wound-induced accumulation of JA is substantially impaired in wounded S-12(T) leaves (Fig. 4), indicating that the production of wound-stimulated JA requires the presence of LOX2. Similar results were observed for the S-13 line, which also lacks LOX2 in leaves of transgenic plants (data not shown).

JA has been proposed to modulate wound-induced gene expression. Therefore, we examined the expression of a wound- and JA-inducible *Arabidopsis* gene, *vsp* (24), in S-12(T) and S-12(C) plants. As shown in Fig. 5, S-12(T) leaves accumulate less vsp mRNA than do S-12(C) leaves in response to wounding. This result suggests that wound-induced JA (or some other LOX2-requiring component of the wound response pathway) is involved in the wound-induced regulation of this gene. The small increase in *vsp* expression in wounded S-12(T) leaves compared to levels found in unwounded leaves could be due to the slight accumulation of JA in these leaves in response to wounding or may be indicative of multiple inputs directing the wound-induced expression of this gene.

DISCUSSION

Although LOX in plants has been extensively studied, our understanding of the physiological significance of LOX activity for specific plant responses is still quite limited. We have chosen to examine this question in *Arabidopsis*, an experimental system that is amenable to genetic manipulation and that appears to have a relatively small LOX gene family. Previous work demonstrated that the two LOX genes thus far identified in *Arabidopsis* differ from each other in sequence and expression pattern (3, 12). In the present report, we demonstrate that one of the encoded proteins, LOX2, is localized in chloroplasts, a potentially important mechanism for compartmentalizing distinct LOX activities.

To identify functions for LOX2, we generated transgenic plants containing sense or antisense constructs of the *Lox2* gene adjacent to a constitutive promoter. One transformed line that has severely reduced expression of LOX2 was characterized in detail to determine the consequences of this loss

Table 1. ABA levels in S-12(C) and S-12(T) plants

Treatment	ABA, μg/g (dry weight) S-12(C)	S-12(T)
Control, 0 hr	1.3 ± 0.8	1.5 ± 1.1
Control, 4 hr	1.3 ± 0.3	2.2 ± 0.8
Wilted, 4 hr	5.5 ± 1.4	5.4 ± 2.0

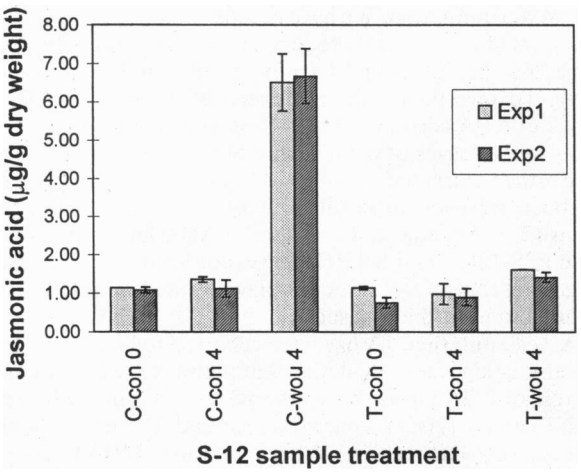

FIG. 4. Analysis of JA abundance in control (C) and transgenic (T) S-12 plants from two experiments. Leaves were collected at the start of the experiment (con 0) or after a 4-h incubation after wounding (wou 4) or no treatment (con 4). Each data point is the average of two or three replicates, with the standard deviation indicated by error bars.

on growth and development and on the accumulation of two growth regulators, JA and ABA.

The apparently normal growth of S-12(T) plants, which contain substantially reduced amounts of LOX2 in both leaves and flowers, indicates that LOX2 levels in control plants exceed those required for normal development. Likewise, no effect on the accumulation of ABA was detected in leaves of plants that are LOX2-deficient. However, S-12(T) leaves fail to accumulate JA in response to wounding. This indicates that LOX2 is required for wound-stimulated JA biosynthesis in leaves and identifies a distinct role for a specific *Lox* gene product in plants.

Chloroplasts have been proposed to be one site of JA synthesis, and the results presented here indicate that a chloroplast LOX is essential for wound-induced JA accumulation in *Arabidopsis* leaves. However, the presence of normal JA levels in unstressed leaves of S-12(T) plants suggests that under normal growth conditions a different LOX is capable of catalyzing JA synthesis. JA has been reported to be transported in phloem (25), suggesting that a nonleaf LOX could provide JA to leaves. The other known

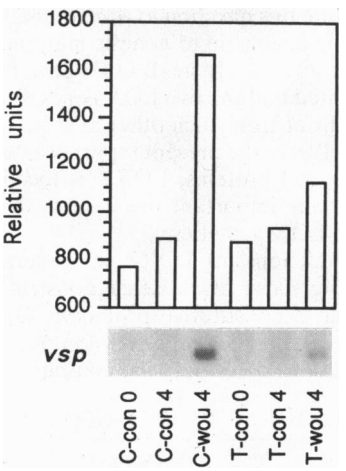

FIG. 5. Analysis of vsp mRNA abundance in leaves of control (C) and transgenic (T) S-12 plants. Leaves were collected at the start of the experiment (con 0) or after a 4-h incubation after wounding (wou 4) or no treatment (con 4). The blot was probed with a *vsp* PCR fragment (24) and quantified by using a Fujix BAS2000 imager.

LOX in *Arabidopsis*, LOX1, is primarily expressed in tissues other than leaves and has no apparent targeting signals (12), suggesting that it is not located in plastids. The capacity of LOX1 to direct JA synthesis remains to be examined. Alternatively, a currently unidentified LOX could direct JA synthesis in unwounded *Arabidopsis* plants or there may be sufficient residual LOX2 in S-12(T) plants to provide the basal JA levels observed.

At present there is still very little known about how JA biosynthesis is regulated in plants, although some characterization of its distribution in various tissues of several species has been done (26). In addition, JA has been shown to accumulate in response to specific stress treatments in leaves (10, 18, 27), etiolated hypocotyls (8), or suspension cultures of various species (9). We expect that further analysis of JA accumulation in plants blocked at a specific point in the JA pathway will provide information about the localization and regulation of JA synthesis in specific growth conditions. The LOX2-deficient plants and others generated by using this experimental approach should also help to identify physiological roles both for LOX and for products of the LOX pathway. For example, the results presented here suggest that wound-induced JA regulates the expression of a wound-induced *Arabidopsis* gene, *vsp*. Investigation of the regulation of additional wound-induced genes in these LOX2-deficient plants should allow us to determine whether wound-stimulated JA is an essential signal modulating wound-induced gene expression. On a broader scale, the analysis of plants with reduced levels of one or more LOXs will provide insight into the involvement of LOX in a number of processes where a role for this enzyme has been proposed, such as defense against insects or pathogens.

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